Developmentally Regulated mRNAs in 3T3-Adipocytes: Analysis of Transcriptional Control

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ABSTRACT We have investigated the regulation of mRNA synthesis during 3T3-adipocyte differentiation by measuring the transcription of specific genes in isolated preadipocyte and adipocyte nuclei. Transcription was assayed by hybridization of newly synthesized RNA to cDNA clones coding for glycerophosphate dehydrogenase (GPD), the induced protein of 13K which is shown here to be related to myelin protein P-2, the induced protein of 28K, actin, and two RNAs that are not developmentally regulated. Transcription of GPD and 13K was observed in adipocyte but not preadipocyte nuclei. Actin was transcribed in both types of nuclei but at a lower level in adipocytes. For most of the RNAs examined, there was a consistent relationship between amounts of nuclear transcription and the abundance of the corresponding cytoplasmic mRNA in adipocytes. However, 13K and 28K mRNAs are 10-100 times more abundant than would be predicted by their nuclear transcription alone. Preliminary mRNA turnover experiments in which 5,6-dichloro-1-D-ribofuranosylbenzimidazole was used to inhibit mRNA synthesis suggest that these mRNAs are much more stable in the adipocyte cytoplasm than the other mRNAs examined. These results indicate that the transcription of specific genes is increased during adipocyte differentiation and suggest that other levels of control, particularly mRNA stability, may contribute to the relative abundance of certain developmentally-regulated mRNAs in adipocytes.

The differentiation of preadipocytes derived from mouse 3T3 cells into adipocytes involves many changes in lipogenic enzyme activities, acquisition of increased hormone sensitivities (reviewed in reference 1), and the production of signals related to tissue angiogenesis (2). These changes in cellular metabolism are accompanied by the synthesis of many new proteins that, together, represent a large fraction of the total adipocyte protein (3, 4). Furthermore, alterations in protein synthesis were shown to correspond to changes in the levels of particular translatable mRNAs (4, 5). More recently, very large alterations in concentrations of several specific mRNAs were demonstrated with cDNA probes isolated from a 3T3-adipocyte clone bank (6). These probes include sequences encoding glycerophosphate dehydrogenase, a key lipogenic enzyme, the abundant previously unidentified proteins of 13K and 28K, and actin.

In the present study, we have examined the mechanisms that regulate the extensive differentiation-dependent changes in mRNA levels by measuring the transcription of specific genes in isolated preadipocyte and adipocyte nuclei. In doing so, we have attempted to determine (a) whether transcription of developmentally-related genes is increased during adipocyte differentiation and (b) whether the levels of transcription correlate with the amounts of these RNAs in adipocytes. The results presented here indicate that transcription of GPD and 13K genes is enhanced during differentiation and suggest that additional levels of control may contribute to the accumulation of 13K and 28K mRNAs.

MATERIALS AND METHODS

Materials: Plasmids containing cDNA inserts of 400, 940, 300, and 1,200 base pairs that are complementary to 13K, 28K, GPD, and actin RNAs, respectively, were described previously (6). Two other plasmids, pC1 and pC2, were selected from a cDNA clone bank as controls for this study because the level of their complementary RNAs changed little upon differentiation. pC1 and pC2 contain inserts of 2.2 and 1.1 kilobases, respectively. DNase I and RNase A were purchased from Worthington Enzymes, Freehold, NJ. RNase T1, proteinase K and all chemicals and enzymes for molecular cloning and DNA sequencing were from Bethesda Research Laboratories (Bethesda, MD).
Poly nucleotide kinase was from New England Biolabs, Beverly, MA. Vanadum
ribonucleoside complexes were prepared as described by Berger and Birken-
meier (7). 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was purchased from
Sigma Chemical Co., St. Louis, MO.

**Cell Culture Conditions:** 3T3-F442A cells (8) were grown in the
Dulbecco-Vogt modification of Eagle's medium supplemented with a mixture of
1% calf serum and 9% cat serum to maintain cells as preadipocytes (9) or with
10% fetal calf serum and 5 μg/ml of insulin to promote the formation of
adipocytes (10). Cells were collected at intervals to which time the number of cells converted to adipocytes had reached a maximum (~80-90%).

**Preadipocyte cultures contained 1-2% adipocytes. Cells were fed 18-24 h
before harvesting.

**Isolation of Nuclei:** Nuclei were isolated from twenty 100-mm cul-
tures of preadipocytes and adipocytes by lysis of cells in 20 μl of 10 mM
Tris-HCl buffer, pH 7.5, containing 5 mM MgCl₂, 0.3 M sucrose, and 0.4% NP-40
(lysis buffer). Nuclei were collected by centrifugation at 1,000 g for 5 min and
were resuspended with 20 μl of 50% glycerol. The nuclear pellet was sus-
pected in 20 μl of 50 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, and 60 μM EDTA at
DNA concentrations of 4.0 to 2.0 μg/ml. The isolation of nuclei was carried out at 4°C. DNA concentrations were measured by the
diphenylamine reaction (10).

In Vitro Transcription: Transcription in nuclei and the isolation of
RNA were performed by modifications of the procedures of McKnight and
Palmiter (11). Nuclei from 10 100-mm cultures of adipocytes or preadipocytes
were incubated at 30°C in 200 μl reaction mixtures containing 20% glycerol,
25 mM Tris-HCl, pH 7.5, 5.5 mM MgCl₂, 0.5 mM MnCl₂, 70 mM KCl, 50
μM EDTA, 2.5 mM dithiothreitol, 5 mM GTP, 5 mM CTP, 10 mM ATP, and
1 μg/ml DNase I. After 20 min, 50 μg of RNA was added, and the reactions were terminated by treatment with DMSO at 20 μg/ml for 5 min. Proteins in the mixtures were then digested for 30 min at 37°C with 100
μg/ml proteinase K in buffer containing 0.1 M Tris-HCl, pH 7.5, 12 mM
EDTA, 0.15 M NaCl, and 1.0% SDS. The reaction mixtures were diluted to
1.0 ml with 10 mM Tris-HCl, pH 7.5, 5.5 mM MgCl₂, passed through a 23 gauge needle,
and precipitated at 4°C with 20 μg/ml of 32P-labeled RNA. The precipitate was dissolved in 50
μl of 1 M sodium acetate, and 2.5 vol of ethanol. The precipitate was dissolved in 50
μl of 1 M sodium acetate and precipitated at 20°C with 2.5 vol of ethanol. The precipitate was dissolved, precipitated with ethanol, and suspended in 100 μl of H₂O.

**Hybridizations of Nuclear Transcripts:** 5 μg of plasmid DNAs
were immobilized on nitrocellulose filters using a Schleicher and Schuell
membrane. Before harvesting. **Isolation of Cytoplasmic RNA and RNA Blotting:**
Cytoplasmic RNA for dot blotting was isolated from the supernatant of the
nuclear isolation (see above). Solid guanidine HCl was added to the cytoplasmic
extracts to give a final concentration of 6 M and the RNA was extracted as
described previously (4, 13). The cytoplasmic RNA was quantitated by absorb-
ance at 260 nm and hybridization to 'H-poly(U). The purified RNAs were
denatured by heating at 60°C with formaldehyde, diluted, and immobilized on
nitrocellulose according to the procedure of White and Bancroft (14). Cyto-
plasmic RNA was isolated from cultures treated with 5,6-dichloro-1-β-D-ribo-
furansylbenzimidazole by the NP-40 lysis procedure (15), electrophoresed on
1.5% agarose gels containing formaldehyde, and transferred to nitrocellulose as
described by Maniatis et al. (15). The nitrocellulose filters were baked at 80°C
under vacuum for 2 h, prehybridized, hybridized to nick-translated probes
(1.2-1.7 × 10⁶ cpm/μg DNA), and washed using the conditions suggested by
Maniatis et al. (15). Autoradiography and quantitation of hybridization signals
by densitometry and liquid scintillation counting were carried out as described
above.

DNA Sequence Determination and Analysis: The 13K plas-
mid used for transcription studies, pAd-5 (6), and an overlapping clone isolated
from our adipocyte cDNA library were used to determine the sequence of the
13K cDNA. Inserts were excised from the plasmids by PsI digestion and
isolated by PAGE. The purified fragments were redigested with EcoRI, SalI or
HhaI and subcloned into the M13 sequencing vectors developed by Messing
(16). Sequencing was done according to the method of Sanger et al. (17) on 80-
cm gels. Both strands of the amino acid coding portion of the cDNAs were
determined. Analysis of the DNA sequence data was done on a Digital 2060
computer with the Inteligencess program or the Lipman-Wilbur programs
(18).

**RESULTS**

**Transcription in Isolated Nuclei**

For our studies of transcription in isolated nuclei, preadipocytes and adipocytes were collected 6 to 7 d after conflu-
ence. This time point was selected because most morpholog-
ical and biochemical changes that accompany adipocyte dif-
ferentiation have reached a maximum by then (4). The time
course of UMP incorporation into transcripts synthesized by
denvedogen RNA polymerases in preadipocyte and adipocyte
nuclei is shown in Fig. 1. Adipocyte and preadipocyte nuclei
show similar patterns of UMP incorporation; incorporation
increases for 30-40 min with half of the total incorporation
occurring in the first 7 min. The initial rates of transcription
for preadipocyte and adipocyte nuclei are ~0.037 and 0.018
pmol/min/μg of DNA, respectively. Adipocyte nuclei appear
to be less transcriptionally active than preadipocyte nuclei
since the activity of adipocyte nuclei per microgram of DNA
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Specific Gene Transcription and RNA Abundance

To assay the transcription of specific genes, 32P-labeled transcripts from preadipocyte and adipocyte nuclei were hybridized to immobilized cDNA probes in identical reaction mixtures (see Materials and Methods). Autoradiograms of the resulting hybridization of nuclear transcripts are shown in Fig. 3. Transcription of GPD and 13K was increased in adipocyte nuclei while no signals for these transcripts were detected in preadipocyte nuclei. The corresponding cytoplasmic RNA dot blots show a large increase in RNA concentration for both of these sequences, although 13K RNA is clearly far more abundant than GPD RNA (Fig. 4). Surprisingly, 28K was not transcribed at detectable levels in either adipocyte or preadipocyte nuclei even though 28K mRNA is very abundant in adipocytes (reference 6 and Fig. 4). Unlike these induced sequences, actin transcripts were synthesized in both types of nuclei although at a lower relative rate in the adipocyte nuclei. Similarly, actin RNA levels decreased during differentiation. Transcription of sequences complementary to the control plasmids, pC1 and pC2, was observed in both types of nuclei. RNA levels for these control clones show little change during differentiation. pC2 is transcribed at a very high level in both adipocyte and preadipocyte nuclei. Transcription of this sequence was not inhibited by α-amanitin at 2 μg/ml (not shown), suggesting that it is not transcribed by RNA polymerase II.

Differentiation-dependent transcriptional control as described above was observed in three separate experiments. Occasionally lower signals were observed with all of the cDNA probes and in these cases, the less abundant nuclear transcripts (13K, GPD, and pC1) were too low to be detected in either adipose or preadipose nuclei. Transcription signals for GPD and 13K were never observed from preadipocyte nuclei.

Quantification of the results shown in Figs. 3 and 4 is presented in Table I, columns A and B. 13K and GPD transcription are increased during differentiation at least 11- and 6-fold, respectively. These are minimum estimates limited by the sensitivity of the assay; the actual enhancement of transcription is likely to be much higher. The differentiation-dependent increases at the RNA level are 41-fold for 13K and at least 17-fold for GPD. Decreases of 1.7 and 1.8-fold were observed, respectively, for actin and actin RNA levels. Transcription of RNA complementary to pC1 and pC2 changes only slightly on differentiation with pC1 decreasing 2.3-fold and pC2 increasing 1.2-fold. The amount of both sequences decreased 1.4-fold at the RNA level.

The correlation between transcription signals and RNA levels for each sequence is reflected in the ratio of these two measurements (Table I, column C). In the cases of the 13K, 28K, and GPD, the ratios could only be calculated for adipocytes since transcription of these sequences was not detectable in preadipocytes.
TABLE I
Specific Nuclear Transcription and Cytoplasmic RNA Levels during Adipocyte Differentiation

<table>
<thead>
<tr>
<th>Probe</th>
<th>Nuclear transcription*</th>
<th>Cytoplasmic RNA levels*</th>
<th>Nuclear transcription/cytoplasmic RNA level*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preadipose</td>
<td>Adipose</td>
<td>-fold change</td>
</tr>
<tr>
<td>13K</td>
<td>--</td>
<td>112</td>
<td>&gt;11†</td>
</tr>
<tr>
<td>28K</td>
<td>--</td>
<td>--</td>
<td>&gt;6†</td>
</tr>
<tr>
<td>GPD</td>
<td>--</td>
<td>62</td>
<td>&gt;6,†</td>
</tr>
<tr>
<td>Actin</td>
<td>824</td>
<td>481</td>
<td>1.7,†</td>
</tr>
<tr>
<td>pC1</td>
<td>91</td>
<td>39</td>
<td>2.3,†</td>
</tr>
<tr>
<td>pC2</td>
<td>8,927</td>
<td>11,145</td>
<td>1.2,†</td>
</tr>
</tbody>
</table>

In vitro nuclear transcription and cytoplasmic RNA dot blots were carried out as described in Materials and Methods. In all cases only those signals >25% above the pBR 322 control were considered positive. Data in columns A and B were derived with the same probes and were not corrected for probe length. A dashed line indicates no detectable positive signal.

* These data were obtained from the experiment shown in Fig. 3. Since extent of hybridization for the different probes was similar (see Materials and Methods), data were not normalized for these values.

† Respective values in column A and column B.

‡ These values were estimated on the basis of the lowest detectable signal (10 cpm for nuclear transcription and 0.75 cpm for cytoplasmic RNA levels).

FIGURE 3
Transcription of specific genes in isolated preadipocyte and adipocyte nuclei. 5.5 x 10⁷ cpm of purified RNA transcribed in nuclei from preadipocytes (P) and adipocytes (A) were hybridized to the immobilized DNAs. After hybridization, the filters were treated with RNase, washed, and exposed to X-ray film as described in Materials and Methods. A resulting autoradiogram is shown. The exposure of pC2 was one seventh as long as that of the other dots.

FIGURE 4
Dot blot analysis of specific RNA levels in preadipocytes (P) and adipocytes (A). (A) 4 μg of immobilized preadipocyte and adipocyte cytoplasmic RNAs were hybridized to cDNA probes nick-translated to specific activities of 1.2-1.7 x 10⁸ cpm/μg. The blots were washed, exposed to X-ray film, and a resulting autoradiogram is shown. For pC2, hybridizations to 0.5 μg of RNA are shown. (B) Hybridization of nick-translated 13K cDNA to dilutions of preadipocyte and adipocyte RNA. The amounts of cytoplasmic RNA in the dots are indicated on the right side of the blot.

TABLE II
Turnover of Cytoplasmic RNAs in Adipocytes

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Apparent half-time of degradation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPD</td>
<td>2</td>
</tr>
<tr>
<td>Actin</td>
<td>4</td>
</tr>
<tr>
<td>13K</td>
<td>12</td>
</tr>
<tr>
<td>28K</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

3T3-F442A adipocytes were maintained 7 d post-confluence in 10% fetal calf serum + 5 μg/ml insulin. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was added to 25 μg/ml in culture medium and cytoplasmic RNA was isolated at 0, 3, 8, 12, 24, and 30 h after the addition of drug. Adipocytes maintained normal morphology and attachment throughout the drug treatment period. The amounts of particular RNA species remaining at various times after the addition of DRB were analyzed by electrophoresing RNA from an equivalent number of cells in formaldehyde/agarose gels, blotting onto nitrocellulose and hybridizing to probes as described in Materials and Methods.

RNA Half-lives in the Presence of DRB

The data presented above suggest that one or more post-transcriptional steps contribute significantly to the accumulation of the 13K and 28K mRNAs. Since the rate of RNA degradation may greatly influence RNA abundance, we have performed a preliminary measurement of RNA half-lives in adipocytes by inhibiting mRNA synthesis with DRB (19, 20) and subsequently measuring loss of these mRNAs by Northern blotting. As shown in Table II, we estimate actin mRNA half-life to be 4 h, while GPD mRNA is 4.7 in the adipocyte. In clear contrast, the ratios for the 13K and 28K protein are 0.23 and <0.06, respectively, reflecting the low transcription signals obtained for these very abundant RNAs.

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of possible secondary effects due to the use of an RNA synthesis inhibitor (21), the results suggest that 13K and 28K mRNAs are very stable in the adipocyte, compared with the other mRNAs examined.

13K Protein Is Closely Related to Myelin Protein P2

The functions of the 13K and 28K proteins are unknown at this time. Since the regulation of 13K and 28K mRNA biosynthesis may be related to the roles of the corresponding proteins in adipocytes, we sought to identify one of the proteins, 13K, by cDNA sequence analysis. Fig. 5 shows 550 bases of the nucleotide sequence obtained from two overlapping cDNA clones and the corresponding derived amino acid sequence. A computer-assisted search of amino acid sequence libraries was performed and three classes of proteins were found to match 13K: P2 myelin proteins, retinoic acid binding proteins, and fatty acid binding proteins. The alignment of the amino acid sequence of 13K with that of rabbit myelin protein P2 (22) (Fig. 6), shows sequence homology of 70% but 80% if conservative amino acid substitutions are allowed. Similarly, a strong homology (48%) is observed between a 32 amino acid fragment of rat testis retinoic acid binding protein (23) and the corresponding portion of the 13K protein (not shown). This result is expected because close amino acid sequence homology between myelin protein P2 and retinoic acid binding protein was described previously (24). Less striking homology, 25% and 47% with conservative substitutions, is observed between the 13K amino acid sequence and that of rat intestinal fatty acid binding protein (25) (Fig. 6). Significantly, myelin protein P2, retinoic acid binding protein, and fatty acid binding protein all appear to interact with lipids (26-28).

DISCUSSION

Our results clearly demonstrate that transcription of GPD and 13K genes in isolated nuclei is greatly increased upon adipocyte differentiation. This is the first demonstration that adipocyte differentiation involves enhanced transcription of specific genes. The transcription of GPD and 13K in adipo-
cytes may be due to either activation of previously untranscribed genes or to enhancement of transcription which, if it occurs in preadipocytes, is below detectable limits. Due to the limited sensitivity of the transcription assays we cannot discriminate between these possibilities. While increased transcription undoubtedly plays a large role in the build up of 13K protein and GPD RNAs in adipocytes, potential differentiation-dependent changes at other levels of regulation cannot be ruled out. Surprisingly, no transcription signal could be obtained for the abundant, strongly differentiation-dependent 28K mRNA (see below). The decrease in actin transcription agrees well with the decrease in actin mRNA observed in these experiments. Although a larger decrease in actin biosynthesis (and presumably mRNA) can be obtained by longer culture and by isolation of pure adipocytes (13), we found the present culture conditions optimal for the routine isolation of active preadipocyte and adipocyte nuclei in good yield. Nuclear transcription results similar to those presented in this study have been observed by M. Phillips, P. Djian, and H. Green (personal communication).

The correlation between relative transcription rate and the steady state abundance differ markedly for particular RNAs in adipocytes (Table I). The ratios of transcription to RNA abundance are similar for GPD, actin, pC1, and pC2. In contrast, 13K and 28K mRNAs are far more abundant than would be expected from their relative transcription rates. It is unlikely that our transcription results reflect some peculiarity of the cDNA probes since the extent of hybridization of RNA fragments to each of these sequences was similar (Materials and Methods). Instead, other steps in mRNA metabolism appear to contribute to the accumulation of 13K and 28K mRNAs. The preliminary results presented in Table II suggest that the abundance of the 13K and 28K mRNAs is due, at least in part, to their turnover times which are long relative to those of GPD and actin. Measurements of the 13K, 28K, and GPD mRNA half lives in preadipocytes are not feasible because of the extremely low abundance of the RNAs in these cells. Hence, our experiments do not indicate whether the apparent stability of the 13K and 28K RNAs is dependent on differentiation or whether it is an intrinsic property of the RNA molecules. Our current experiments are aimed at determining the half-lives of these mRNAs more precisely and defining the factors that control mRNA stability in preadipocytes and adipocytes.

Many studies have previously demonstrated developmentally related changes in specific gene transcription in other systems (reviewed in reference 29). While a good quantitative correlation is generally observed between relative transcription levels and RNA abundance (30) there are now several examples of mRNAs whose abundance cannot be accounted
for by relative transcription levels alone (31–33). In most of these cases, the mRNA species have an intrinsically long half-life or a developmentally-related increase in specific mRNA half-life (29, 31, 32). In adipocytes, what physiological consequences could arise from the different contributions made by transcription and RNA turnover to the abundance of 13K and 28K and GPD mRNAs? An mRNA which is transcribed relatively rapidly and degraded rapidly, such as GPD, could be quickly removed by stopping transcription when its presence is deleterious and later quickly restored by reactivating transcription. This control is probably useful to the adipocyte because while glycerol-3-phosphate, the product of GPD, is required in large amounts when cells are in a lipogenic mode, removal of this molecule during lipolysis may minimize reesterification of the newly formed free fatty acids. While the physiological role for 13K has not been determined, it is likely to function as a lipid binding protein because it is related by amino acid sequence to three proteins that interact with lipids: myelin protein P2, retinoic acid binding protein and fatty acid binding protein (Figs. 5 and 6, 26–28). The sequence of an adipocyte mRNA related to myelin protein P2 has also been recently described by Bernlohr et al. (34). Since fatty acids and triglycerides are very abundant when adipocytes are in either lipogenic or lipolytic modes, rapid regulation lipid–binding proteins may not be necessary. Some support for this rationale is the decreased synthesis previously observed in GPD and fatty acid synthetase but not in 13K protein when differentiating adipocytes were challenged with drugs which raise the concentration of intracellular cyclic AMP, a key lipolytic agent (5, 35). It is likely that the ability of the adipocyte to meet its multiple metabolic roles will require several regulatory mechanisms for different mRNAs.

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